Morphine induces Beclin 1- and ATG5 dependent autophagy in human neuroblastoma SH-SY5Y cells and in the rat hippocampus

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Abbreviations: EGFP, enhanced green fluorescent protein; LC3, microtubule-associated protein light chain 3; BAF A1, bafilomycin A1; PTX, pertussis toxin; ATG5, autophagy related gene 5; ATG7, autophagy related gene 7; Bcl-2, B-cell lymphoma/leukemia-2; Bcl-xL, B-cell lymphoma extra long; Co-IP, co-immunoprecipitation; EBSS, earle’s balanced salt solutions

Chronic exposure to morphine can induce drug addiction and neural injury, but the exact mechanism is not fully understood. Here we show that morphine induces autophagy in neuroblastoma SH-SY5Y cells and in the rat hippocampus. Pharmacological approach shows that this effect appears to be mediated by PTX-sensitive G protein-coupled receptors signaling cascade. Morphine increases Beclin 1 expression and reduces the interaction between Beclin 1 and Bcl-2, thus releasing Beclin 1 for its pro-autophagic activity. Bcl-2 overexpression inhibits morphine-induced autophagy, whereas knockdown of Beclin 1 or knockout of ATG5 prevents morphine-induced autophagy. In addition, chronic treatment with morphine induces cell death, which is increased by autophagy inhibition through Beclin 1 RNAi. Our data are the first to reveal that Beclin 1 and ATG5 play key roles in morphine-induced autophagy, which may contribute to morphine-induced neuronal injury.

Introduction

Morphine is clinically used for pain relief in cancer patients. However, chronic exposure of morphine can induce drug addiction, gross impairment of dopaminergic neurons and neural injury.1 Indeed, numerous reports show that morphine induces brain damage and neuronal toxicity by inhibiting cell growth and inducing apoptosis both in vitro and in vivo.2,3 These inhibitory effects may be G protein-dependent following the engagement of morphine with the opioid receptors in SK-N-SH cells.4 Morphine promotes apoptosis in macrophages and in Jurkat T-cells through oxidative stress, which subsequently activates cell death pathways,5 or through production of TGFβ,6 which may contribute its effect on immune suppression. In contrast to these reports, there are also studies showing that morphine can have protective effects against cell death.10,11 Morphine prevents peroxynitrite-induced death of SH-SYSY cells through a direct scavenging action,12 and it even stimulates cell growth in mouse retinal endothelial cells.13 It appears that the effects of morphine on cell death are cell type-dependent, and the exact mechanism of morphine-induced neurotoxicity remains a subject of debate.

Autophagy is a regulated cellular degradative pathway that involves the delivery of cytoplasmic cargo to the lysosomes. In neurons, a constitutive, basal level of autophagy helps to control the cellular quality of proteins14 and protects cells from protein aggregation15 or damaged organelles.16 Autophagy can be activated in response to environmental cues such as nutrient depletion and temperature and oxidative stresses.17 Autophagy is highly regulated by the ATG genes such as Beclin 1,18 ATG5,19 and ATG7.20 Beclin 1 is a phylogenetically conserved protein that is essential for the initiation of autophagy.21 Beclin 1 interacts with numerous partners such as UVRAG, Ambral and Bif-1 in the initiation of autophagosome formation.22 This process is strongly inhibited by Bcl-2 and Bcl-xL, the key anti-apoptotic Bcl-2 family proteins.23 The interaction of Beclin 1 with Bcl-2 plays a key role for the regulation of autophagy in addition to Bcl-2’s established role in apoptosis.24 In contrast to its protective effect, autophagy can also be a cell death mechanism.25 Ischemia/hypoxia,26 oxidative stress27 and some chemical reagents, such as methamphetamine,28 tryptamine29 and dopamine,30 induce autophagic cell death in neuronal cell lines or normal neurons. We herein address the possibility that morphine may activate autophagy which may
result in autophagy-associated cell death of neuronal cells. Our data are the first to reveal that Beclin 1 and ATG5 play key roles in morphine-induced autophagy. Better understanding of the mechanisms of action of morphine holds promise for better management of cancer patients and morphine use.

**Results**

During autophagy, the cytoplasmic form of LC3 (LC3-I, 18 kDa) is converted to the preautophagosomal and autophagosomal membrane-bound form (LC3-II, 16 kDa). LC3 is thus used as a specific marker for autophagosome formation, although there are some limitations as it is tissue- and cell-dependent. Transient overexpression of GFP-LC3 is not used as LC3 aggregates are often formed within cells. To determine whether morphine could induce autophagy in neuronal cells, we treated the pEGFP-LC3 stably transfected SH-SY5Y cells with morphine hydrochloride and found that there were increased numbers of punctate GFP-LC3 dots in the treated cells in a time- and dose-dependent manner. Western blotting analysis revealed a steadily increasing quantity of the LC3-II form in morphine-treated SH-SY5Y cells. As increased LC3-II levels can occur when autophagy is either induced or inhibited, lysosomal inhibitor bafilomycin A1 (BAF A1), which prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes, was used to determine that morphine-induced LC3-II levels increased as a result of increased autophagosome formation rather than a defect in the fusion process. Results showed that BAF A1, significantly increased LC3-II levels (Fig. 1C and D) and autophagosome formation (Fig. 1B). Autophagy is characterized by the formation of double-membraned autophagosomes that fuse with lysosomes and undergo degradation. To confirm that morphine induces autophagy, we examined the ultrastructure of the cells by electron microscopy and found abundant vacuolar elements which are most likely to be of autophagic origin in SH-SY5Y cells after treatment with 200 µM morphine for 24 h (Fig. 1E). Collectively, our results clearly demonstrated that morphine induces an autophagic response in SH-SY5Y cells.

Morphine activates a receptor-mediated G protein-coupled signaling pathway upon engagement with its receptors. We therefore asked whether morphine-induced autophagy is mediated by opioid receptors. We first used a pharmacological approach to address this question. Naloxone is a general antagonist of opioid receptors, and previous reports showed that it blocks the effects of morphine. Our results show that pretreatment of SH-SY5Y cells with naloxone (100 µM) reduced the morphine-induced increase in LC3-II levels (Fig. 2A). We next used PTX and suramin, two antagonists of G protein signaling pathways, and found that both PTX (100 ng/ml) and suramin (100 µM) completely blocked the increase in LC3-II levels (Fig. 2B). These results provide strong evidence to suggest that morphine induces autophagy through an opioid receptor-mediated and PTX-sensitive G protein pathway.

Beclin 1 is one of the key mediators in the formation of the autophagosome, as it is involved in the initial step of autophagosome formation. We hypothesized that Beclin 1 is also important for morphine-induced autophagy. Consistent with this hypothesis, we found that Beclin 1 is upregulated in morphine-treated SH-SY5Y cells (Fig. 3A). In contrast, the expression of Bcl-2, Bcl-xL, two Beclin 1-interacting proteins in the Bcl-2 family, were not affected. The pro-apoptotic Bcl-2 protein Bax, also remained unchanged. It has been reported that Beclin 1 is released from Bcl-2 during the initiation of autophagy. We therefore determined whether morphine disrupts the interaction between Beclin 1 and Bcl-2, thereby initiating autophagy. Indeed, the results of co-immunoprecipitation (Co-IP) with anti-Bcl-2 antibody and blotting with anti-Bcl-2 and anti-Beclin 1 antibodies showed that the interaction of Beclin 1 with Bcl-2 progressively decreased (Fig. 3B and C). To further substantiate the role of Bcl-2 in the regulation of autophagy, we found that Bcl-2 overexpression significantly reduced the morphine-induced increase in LC3-II levels (Fig. 3D) and increased the binding with Beclin 1 (Fig. 3E). To confirm the function of Beclin 1 in morphine-induced autophagy, we used a shRNA expressed in the pSilencer 2.1-U6 Hygro vector, which specifically knocked down Beclin 1 expression in SH-SY5Y cells. As expected, the level of autophagy induced by morphine, as shown by LC3-II levels, was decreased by Beclin 1 suppression (Fig. 3F). Collectively, these data demonstrate that morphine induces Beclin-1 release from Bcl-2 and that the released Beclin 1 plays a key role in morphine-induced autophagy.

In addition to Beclin 1, ATG5 has been reported to regulate autophagy; therefore, we next examined ATG5’s role in morphine-induced autophagy. We found that morphine completely failed to induce autophagy in ATG5-knockout MEFs, whereas wild-type MEFs showed pronounced autophagy upon morphine treatment, although less than that in SH-SY5Y cells (Fig. 4A). Unlike Beclin 1, however, the level of ATG5 protein remained unchanged with morphine treatment (Fig. 4B). These results showed that ATG5 is also important in morphine-induced autophagy.

All the data clearly show that morphine induces autophagy in SH-SY5Y cells. We next asked whether morphine might induce autophagy in the rat brain. Our results showed that either chronic or acute morphine treatment increased LC3-II levels in the hippocampus; however, we found no detectable effect of morphine on LC3-II levels in the striatum (Fig. 5A). This result indicates that morphine-induced autophagy may be cell-type specific in the brain. Naloxone also inhibited the morphine-induced increase in LC3-II levels in the rat hippocampus (Fig. 5B), confirming that morphine-induced autophagy is mediated through the opioid receptors.

Accumulating evidence has shown that autophagy has dual roles in cell death. Some results suggest that autophagy in neurons provides a neuroprotective mechanism, however, some reports show that autophagy is harmful. Increasing evidence suggests that the effects of autophagy are highly contextual. We therefore asked whether morphine-induced autophagy has a protective or a harmful role in the neural system. Cell death detection showed that higher dose (500, 1,000 µM at 48 h) or longer time (200 µM at 72 h) of morphine treatment could induce cell death (Fig. 6A), which was increased when...
Figure 1. For figure legend, see page 4.
addiction and neural injury. The exact mechanism is not fully understood. The present study revealed that the exposure to morphine induces autophagy in SH-SY5Y cells and in the rat hippocampus. Autophagy is an early event following the treatment of morphine; it is found that autophagy could be detected as early as 0.5 h with morphine treatment (Fig. S1B). Accumulating evidence suggests that autophagy-associated cell death or type II programmed cell death,41 can occur in some cell types, but the role of autophagy in morphine-induced cell death was not previously explored. We detected cell death (Fig. 6) in SH-SY5Y cells at later time points and cell death was increased by autophagy inhibition by Beclin 1 knockdown (Fig. 6B and C). The results that cell death was increased by autophagy inhibition indicated that autophagy is an early response to the morphine induced-stress and may have a protective role in cell death; however, chronic exposure leads to extensive autophagy which may damage the cellular components leading towards cell death.

**Discussion**

Morphine is widely used clinically for pain management in cancer patients. Chronic exposure of morphine could induce drug addiction and neural injury. The exact mechanism is not fully understood. The present study revealed that the exposure to morphine induces autophagy in SH-SY5Y cells and in the rat hippocampus. Autophagy is an early event following the treatment of morphine; it is found that autophagy could be detected as early as 0.5 h with morphine treatment (Fig. S1B). Accumulating evidence suggests that autophagy-associated cell death or type II programmed cell death,41 can occur in some cell types, but the role of autophagy in morphine-induced cell death was not previously explored. We detected cell death (Fig. 6) in SH-SY5Y cells at later time points and cell death was increased by autophagy inhibition by Beclin 1 knockdown (Fig. 6B and C). The results that cell death was increased by autophagy inhibition indicated that autophagy is an early response to the morphine induced-stress and may have a protective role in cell death; however, chronic exposure leads to extensive autophagy which may damage the cellular components leading towards cell death.

**Figure 1 (See previous page).** Morphine induces autophagy in SH-SY5Y cells, in a time- and dose-dependent manner, and is increased by bafilomycin A1. (A) Punctate GFP-LC3 dots in morphine-treated SH-SY5Y cells. pEGFP-LC3 stably transfected SH-SY5Y cells were treated with 200 µM morphine for the indicated times. Cells were fixed with formaldehyde (3.7% w/v) and immunostained with anti-Lamp-3 for detecting lysosomes. Cells were examined by fluorescence confocal microscopy (X63/0.15). (B) pEGFP-LC3 stably transfected SH-SY5Y cells were treated with 200 µM morphine for the indicated times, with or without 20 nM BAF A1. Punctate GFP-LC3 dots in cells were counted. Data were the mean value of three independent experiments with each count of no less than 200 cells. *p < 0.01 as compared with control. **p < 0.001 as compared with morphine alone. (C and D) LC3-II was significantly increased in morphine-treated SH-SY5Y cells, and was increased by BAF A1. (C) SH-SY5Y cells were exposed to 100 or 200 µM morphine for 12 h, with or without 20 nM BAF A1 and then subjected to western blotting analysis with anti-LC3 antibody. Positions of LC3-I and LC3-II are indicated. The data quantified by Image J software are shown on the top of the panel. Results were shown as average ± SD for three separate experiments. *p < 0.001 as compared with control. **p < 0.001 as compared with morphine alone. (D) SH-SY5Y cells treated with 200 µM morphine for the indicated times, with or without 20 nM BAF A1 were also subjected to western blotting analysis. The data quantified by Image J software are shown on the top of the panel. Results were shown as average ± SD for three separate experiments. *p < 0.01 as compared with control. **p < 0.01 as compared with morphine alone. (E) Electron micrographs showing the ultrastructure of morphine-treated SH-SY5Y cells. (a) Control (untreated SH-SY5Y cells), (b and c) SH-SY5Y cells treated with 200 µM morphine for 24 h. Arrows in the electron micrograph denote representative presumed autophagic bodies.

**Figure 2.** Morphine induces autophagy through an opioid receptor-mediated PTX-sensitive G protein pathway. (A) SH-SY5Y cells were treated with or without 100 µM naloxone for 30 minutes and then treated with different concentrations of morphine for 12 h. LC3-I and LC3-II were detected by western blotting analysis. The data quantified by Image J software are shown on the top of the panel. Results were shown as average ± SD for three separate experiments. *p < 0.001 as compared with control. **p < 0.001 as compared with morphine alone. (B) SH-SY5Y cells were treated with or without 100 ng/ml PTX or 100 µM suramin for 30 minutes, after which 200 µM morphine was added for 12 h. LC3-I and LC3-II were detected by western blotting analysis. The data quantified by Image J software are shown on the top of the panel. Results were shown as average ± SD for three separate experiments. *p < 0.001 as compared with control. **p < 0.001 as compared with morphine alone.
It is well recognized that morphine could bind to opioid receptors leading to the activation of the G protein-coupled receptor mediated pathway. Our results showed that autophagy induced by morphine is mediated by opioid receptors in a G protein-dependent manner. We showed that naloxone, a general antagonist of opioid receptors, and PTX and suramin, two G protein-coupled receptor antagonists, strongly inhibited morphine-induced inhibition by knockdown of Beclin 1. These data suggest that autophagy is an early response to the morphine induced-stress and may have a protective role in cell death; however, chronic exposure leads to extensive autophagy which may damage the cellular components leading towards cell death. All of these results may help to explain how chronic exposure of morphine may contribute to neural injury.
JNK, which can be activated by a G protein signaling pathway, to the increase of Beclin 1. Also, we considered the possibility that reduces autophagy in SH-SY5Y cells. It would be interesting to autophagy. In addition, knockdown of Beclin 1 by shRNA morphine, and Bcl-2 overexpression blocks morphine-induced between Beclin 1 and Bcl-2 is reduced following treatment with treatment with morphine. Supporting this, we found that the interaction between Beclin 1 and Bcl-2 may regulate autophagy induced by morphine. The exact mechanism through which the G protein-coupled signaling pathway is linked with autophagic machinery needs to be further elucidated. Our data revealed that the interaction between Beclin 1 and Bcl-2 may regulate autophagy induced by morphine. Supporting this, we found that the interaction between Beclin 1 and Bcl-2 is reduced following treatment with morphine, and Bcl-2 overexpression blocks morphine-induced autophagy. In addition, knockdown of Beclin 1 by shRNA reduces autophagy in SH-SY5Y cells. It would be interesting to examine if the activation of G protein pathway is directly related to the increase of Beclin 1. Also, we considered the possibility that JNK, which can be activated by a G protein signaling pathway, can phosphorylate Bcl-2 and thereby attenuate the interaction of Beclin 1 and Bcl-2, enhancing autophagy. However, the JNK inhibitor SP600125 appears not to prevent morphine-induced autophagy in SH-SY5Y cells. We found that ROS production is increased in morphine-treated SH-SY5Y cells and that the ROS scavenger, NAC, blocks ROS production and morphine-induced autophagy (Fig. S3). Thus, the increase of ROS induced by morphine may promote autophagy.

It is interesting to note that LC3-II increase is detected in the hippocampus but not in other regions of the brain. Our study provides evidence that morphine exerts its toxic effects through the induction of autophagy in the rat brain. The results are consistent with a previous study which shows that chronic exposure to morphine dramatically alters neuronal phenotypes in the dentate gyrus-CA3 region of the adult rat hippocampus, although autophagy was not documented in this study. Why the hippocampus region is the most affected region in the brain remains to be determined. It has been reported that a high dose of dopamine induces autophagic cell death in SH-SY5Y cells. There may be interplay between the morphine-activated signaling pathway and dopamine-related signaling pathways for autophagic cell death. Hypoxia-ischemic injury induces a dramatic increase in autophagosome formation and extensive hippocampus neuron death, but mice deficient in Atg7 are defective for autophagy and neuron death, suggesting that autophagy is causally linked with cell death. Further work will address how morphine leads to neuronal cell death in the absence of ATG7 or other ATG genes.

**Materials and Methods**

**Materials.** Morphine hydrochloride was purchased from Qinghai Company, China. pEGFP-C1-LC3 plasmid was kindly provided by Dr. Noboru Mizushima (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). The rabbit anti-LC3 polyclonal antibody was kindly provided by Dr. Tamotsu Yoshimori (National Institute of Genetics, Shizuoka-ken, JAPAN) and Dr. Yingyu Chen (Peking University Health Science Center, Beijing, China) and was purchased from Sigma (L7943). Anti-β-actin (monoclonal, A5316) and anti-ATG5 (polyclonal, A0856) antibodies were purchased from Sigma. Anti-Beclin 1 (monoclonal, 612113) and anti-Bcl-2 (monoclonal, 610539) antibodies were purchased from BD Transduction Labs. Anti-Bcl-xL (polyclonal, 56361) antibody was purchased from BD Pharmingen. Anti-Bax (polyclonal, sc-493) antibody was purchased from Santa Cruz Biotechnology. Secondary antibodies (HRP-labeled Goat Anti-Mouse IgG, 074-1806; HRP-labeled Goat Anti-Rabbit IgG, 074-1506) were purchased from KPL, Kirkegaard & Perry Laboratories. Enhanced chemiluminescence (ECL) reagents (WBKLS0500) were purchased from Millipore. All other chemicals were purchased from Sigma unless otherwise specified.

**Cell culture, transfection and beclin 1 RNAi.** SH-SY5Y, wild-type and ATG5-knockout mouse embryonic fibroblast (MEF) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, 12100-046; Gibco-BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS, SH30088.03; Thermo Scientific HyClone), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. Cells were maintained in a humidified 10% CO2 atmosphere at 37°C. SH-SY5Y cells at subconfluence were transfected with pEGFP-C1-LC3 plasmid DNA using Lipofectamine™ 2000 Reagent (11668-019, Invitrogen Corporation), following the procedure recommended by the manufacturer and then selected with 600 µg/ml geneticin. A human Beclin 1 shRNA hairpin (target sequence: CTC AGG AGA GGA GCC ATT T) was cloned into the HindIII and BamH1 sites of pSilencer 2.1-U6 Hygro. Beclin 1 shRNA and control scrambled shRNA plasmids were transfected into SH-SY5Y cells and selected with 50 µg/ml Hygromycin.

**Fluorescence confocal microscopy.** pEGFP-LC3 stably transfected SH-SY5Y cells were cultured on coverslips and then treated with or without morphine for the indicated times. The coverslips
analysis was performed to monitor the red fluorescence of DNA-bound PI (630 ± 22 nm). All data were analyzed with Cell Quest software (BD).

**Morphine treatments.** Rats were divided into four groups (4 rats in each group) and injected subcutaneously with saline or 10 mg/kg morphine hydrochloride for 9 days. The animals received 18 injections of saline (control) or morphine (“chronic” group), 17 injections of saline preceding an injection of morphine.
four groups above were sacrificed 2 h after the last injection, and injection of morphine (“acute + naloxone” group). Animals in the 170 mg/kg naloxone (Sigma-Aldrich, USA) 30 min before the last (“acute” group), or 17 injections of saline with an injection of 5 mg/kg saline (“control” group) or 17 injections of saline with an injection of 5 mg/kg morphine (30770719) to N. Sui.

**Figure 6.** Morphine induces cell death, which is increased by autophagy inhibition. (A) SH-SYSY cells were treated with 0, 200, 500, 1,000 µM morphine for 48, 72 h, then cells were harvested and stained with PI, detected by flow cytometry analysis. The amounts of PI positive cells were quantified. Results were shown as average ± SD for three separate experiments. *p < 0.05, **p < 0.01 compared with control. (B) Beclin 1 shRNA or scrambled shRNA SH-SYSY cells were treated with or without 200 µM morphine for 72 h, then cells were harvested and stained with PI, detected by flow cytometry analysis. The amounts of PI positive cells were quantified. Results were shown as average ± SD for three separate experiments. *p < 0.01 compared with control. *p < 0.01 compared with scrambled shRNA SH-SYSY. (C) Beclin 1 shRNA or scrambled shRNA SH-SYSY cells were treated with or without 200 µM morphine for 72 h, then cells were harvested and lysed with lysis buffer, Beclin 1, LC3-I and II levels were detected with anti-LC3 and anti-Beclin 1 antibodies.

(“acute” group), or 17 injections of saline with an injection of 5 mg/kg naloxone (Sigma-Aldrich, USA) 30 min before the last injection of morphine (“acute + naloxone” group). Animals in the four groups above were sacrificed 2 h after the last injection, and the hippocampuses and striatums were isolated and prepared for western blotting analysis. The experimental protocol and procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

**Statistical analysis.** Data were analyzed as means ± SD. The data were evaluated statistically by the analysis of variance (ANOVA). Significance was determined as p < 0.05.

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**Supplementary materials can be found at:** www.landesbioscience.com/supplement/ZhaoAUTO6-3-Sup. pdf

**References.**


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